MEDIATING INTERNALIN A-DEPENDENT ENTRY OF MICROSPHERES IN EPITHELIAL CELLS

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MEDIATING INTERNALIN A-DEPENDENT ENTRY OF MICROSPHERES IN EPITHELIAL CELLS

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{280}	Absorbance at 280 nm
A_{562}	Absorbance at 562 nm
A_{600}	Absorbance at 600 nm
DTT	Diothreithol
E-cadherin	Epithelial cadherin
FITC	Fluorescein isothiocyanate dye
FITC-OVA	Ovalbumin labeled with FITC dye
GST	Glutathione S-transferase
InlA	Internalin A protein
IPTG	isopropyl β-D-1-thiogalactopyranoside
L. monocytogenes	*Listeria monocytogenes*
pGEX	pGEX-6P-1-InlA-1 plasmid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SUMMARY

Internalin A, an internalin protein found in the food-borne pathogen *Listeria monocytogenes,* allows the pathogen to enter host cells through receptor-mediated internalization. Through Internalin A-mediated entry, *L. monocytogenes* invades enterocytes by binding to the receptor protein E-cadherin (Bergmann et al 2002). In this way, the pathogen is able to cross the intestinal barrier, a highly selective permeable interface that is responsible for allowing nutrients into the intestinal lumen while barring the entry of waste and pathogens. This study seeks to observe Internalin A-mediated entry of a pathogen mimetic system into epithelial cells. We use polystyrene carboxyl-terminated microspheres to display Internalin A, study the effect on internalization of ligand density and the size of the microsphere. A pGEX plasmid containing the *inlA* gene, which had previously been purified after transformation into MAX Efficiency DH5αF'IQ *E. coli* competent cells, was transformed into and expressed in OneShot BL21(DE3)pLysS *E.coli* competent cells. The result of expression of the plasmid was the Internalin A protein (InlA), combined with a glutathione S-transferase (GST) tag, in order to form a 75 kDa InlA-GST fusion protein. This fusion protein was subsequently purified through affinity chromatography. Concurrently, a protocol for labeling protein with fluorescein isothiocyanate dye (FITC) and covalently coupling the protein to 2 μm microspheres was also developed using ovalbumin. The future steps in this experiment are to successfully cleave the GST tag from Internalin A using sequence-specific protease, functionalize microspheres with purified InlA labeled with FITC and perform internalization studies with microspheres of different sizes and different densities of protein coating. Because InlA can effectively facilitate transport of *L. monocytogenes*

into the cells of the intestinal epithelium, this study has important implications for improving the efficiency of drug delivery to the intestinal lumen.

CHAPTER 1

INTRODUCTION

Receptor-mediated internalization is a common mechanism used by intracellular pathogens to gain entry into host cells. Receptor proteins found on host cells play a crucial role in mediating cell signaling, cell growth and cell division. Binding of a ligand to a cellular receptor protein initiates signaling pathways in the cell, resulting in changes in cellular activity (Alberts and Johnson, 2002). Ligand binding also plays a key role in modulating immune response in host cells, commonly seen in phagocytosis. After binding of pathogen-derived ligands to receptors on the surface of phagocytotic cells, these cells form a vesicle called a phagosome that engulfs the pathogen. Maturation and fusion of the phagosome with the lysosome triggers acidification of the phagolysosome, which activates degradative enzymes in the lysosome and thus destroys the pathogen (Alberts and Johnson, 2002). While the mechanism and time scale of the steps of phagocytosis have been studied extensively in "professional" phagocytotic cells, such as macrophages, this process is not well understood in "non-professional" phagocytotic cells (Blanchette et al, 2009).

After binding of a pathogen-derived protein to a cell surface receptor, the pathogen is able to enter the cell. Immediately after entry, the pathogen is surrounded by a phagocytic vacuole that will eventually degrade the pathogen through acidification. However, through the concerted action of pathogen enzymes and proteins derived from the host cell, the pathogen can then escape the phagocytic vacuole and polymerize the free actin of the host cell, thus migrating effectively between cells (Pizarro-Cerda and

Cossart, 2006). *Listeria monocytogenes* is a prime example of a pathogen that effectively invades host cells by binding to cell surface receptors. Internalin A, a protein found on the cell surface of *L. monocytogenes*, binds to epithelial cadherin (E-cadherin), a cell surface receptor found on epithelial cells. In this way, Internalin A is able to invade enterocytes in humans and then polymerize free actin of the host cells to migrate between cells (Bergmann et al 2002). In order to effectively treat diseases caused by intracellular pathogens, it is imperative to understand the mechanism of entry of the pathogen. This knowledge can thus be applied to develop a mode of drug delivery that effectively facilitates entry of the drug into cells.

In Dr. Todd Sulchek's lab, I have expressed and attempted to purify the InlA-GST fusion protein from a pGEX-6P-1-InlA-1 plasmid obtained from Dr. Youn-Hi Woo's lab. This plasmid was previously transformed into and purified from MAX Efficiency DH5αF'IQ competent *E. coli* cells (Invitrogen, Carlsbad, CA). The purified plasmid was transformed into OneShot BL21(DE3)pLysS competent *E. coli* cells (Invitrogen, Carlsbad, CA). Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) in transformed BL21(DE3)pLysS cell cultures. The use of BL21(DE3)pLysS cells allowed me to induce transcription because of the nature of the DE3 lysogen in these cells. The DE3 lysogen carries a gene for T7 RNA polymerase. T7 RNA polymerase is under control of the *lac*UV5 promoter, whose transcription is inducible with the addition of IPTG (Invitrogen, 2010). Absorbance readings were taken to determine the growth phase of the cell cultures, and transcription of T7 RNA polymerase was thus induced when the cell cultures reached the mid-log phase of growth.

In this way, protein yield could be maximized and transcription was induced in healthy and viable cells.

Protein was subsequently purified through affinity chromatography. I used Glutathione Spin Columns (Pierce, Rockford, IL) because of GST's high binding affinity for glutathione (Blanchette et al, 2009). The InlA-GST protein was subsequently displaced from the column with excess reduced glutathione, leading to elution of the fusion protein from the column (ThermoScientific, 2010). A BCA assay was used to quantify the concentration of protein in solution. Cu^{2+} ions are reduced to Cu^{1+} ions through the Biuret reaction, in which copper is chelated with protein in an alkaline medium (ThermoScientific, 2010). The colorimetric nature of the BCA assay is dependent on the detection of Cu^{1+} ions by bicinchoninic acid. In order to account for the presence of reducing agents, such as diothreithol and glutathione, in the InlA-GST fusion protein solution, a Reducing Agent Compatible Microplate BCA Assay Kit (Pierce, Rockford, IL) was used.

I have also concurrently labeled ovalbumin with FITC and functionalized 2 μm polystyrene carboxyl-terminated microspheres with FITC-labeled ovalbumin (FITC-OVA). These functionalized microbeads can be used as a negative control in future steps of the experiment, when performing internalization assays with InlA-functionalized microbeads. In addition, I chose to use ovalbumin when developing the protocol for the functionalization of microbeads because ovalbumin has a molecular weight of 45 kDa. This is comparable to the molecular weight of InlA, which is 50 kDa, meaning that microspheres have similar binding capacities for both of these proteins and the same mass of protein may be used to effectively functionalize the microspheres. This makes it

easy to use the same protocol developed for binding FITC-OVA to microspheres when functionalizing the microspheres with InlA.

Future steps in this experiment include cleaving the GST tag from the fusion protein with protease, labeling the purified InlA with FITC and covalently coupling the labeled protein to microspheres. After this, internalization assays will be conducted with Caco-2 cells using flow cytometry and fluorescence microscopy in order to observe the effect of InlA functionalization, bead size and density of protein bound the microspheres on microsphere internalization.

CHAPTER 2

BACKGROUND INFORMATION AND LITERATURE REVIEW

Cells rely heavily on cell surface receptor proteins to regulate cell signaling, cell growth and cell division. Binding of a ligand to a cell surface receptor typically elicits changes in the cell and commonly initiates signal transduction pathways in the cell. In this way, factors of the extracellular environment can modulate gene expression and intracellular protein activity. A prime example of a cell surface receptor is epithelial cadherin, or E-cadherin. E-cadherin is a cell adhesion molecule (CAM) that regulates Ca^{2+} adhesion of adjacent cells, playing a crucial role in cell signaling and embryonic development (Alberts and Johnson, 2002).

Because of the high affinity of ligands for particular cell surface receptors, receptor proteins also play a key role in recognition of pathogens and initiation of appropriate immune responses, such as phagocytosis. After binding of pathogen-derived ligands to receptors on the surface of phagocytotic cells, these cells form a vesicle, called a phagosome, which engulfs the pathogen (Cooper, 2000). Maturation and fusion of the phagosome with the lysosome triggers acidification of the phagolysosome, which activates degradative enzymes in the lysosome and thus destroys the pathogen (Cooper, 2000). This mechanism is has been studied and is well understood in cells whose primary function is phagocytosis (e.g. macrophages and neutrophils), or "professional" phagocytes. However, phagocytosis also occurs as a secondary function in other cells, called "nonprofessional" phagocytes, and these cells are more limited in the types of molecules that they can engulf. It is in these cells that receptor-mediated internalization of intracellular pathogens commonly occurs. It is known that intracellular pathogens

such as *Yersinia enterocolitica* and *Listeria monocytogenes* are not only able to effectively invade host cells through binding of surface proteins to host cell surface receptors, but are also able to evade host immune responses (Bogdan, 2008). Although the process of invading host cells is an energetically demanding process for the pathogen, the pathogen is able to safely and successfully reproduce inside the host cell and keep its metabolic demands to a minimum by parasitizing the host cell (Bogdan, 2008).

L. monocytogenes, a Gram-positive bacteria and food-borne pathogen commonly found in produce and undercooked meat, invades cells through host cell surface receptor interaction with a family of proteins called internalins. The internalin family of proteins is characterized by binding of the protein to the cell surface receptor of the host through leucine-rich repeat domains. The two best-understood mechanisms of internalization of *L. monocytogenes* in mammalian cells are mediated by Internalin A and by Internalin B (Lecuit, 2005). These two surface proteins interact with different host cell surface receptors in order to promote entry of *L. monocytogenes.* Internalin B binds to the Met receptor, a tyrosine kinase receptor located on hepatocytes. Thus, Internalin B-mediated listeriosis manifests itself as liver infection (Gaillard et al., 1991). Internalin A, on the other hand, binds to E-cadherin in intestinal epithelial cells facilitating entry of the pathogen into the intestinal lumen. After gaining entry to host cells, *L. monocytogenes* is engulfed in a phagocytotic vacuole, but is able to escape through expression of the protein listeriolysin. After successful evasion of the host cell's immune response, *L. monocytogenes* migrates between cells by polymerizing the host cell's free actin through expression of the actA protein, thus allowing the pathogen to cross biological barriers (Lecuit, 2005). In this way, *L. monocytogenes* is capable of traversing the fetoplacental

barrier, possibly leading to spontaneous abortion in infected pregnant women. In the most serious cases of listeriosis, *L. monocytogenes* can cross the blood-brain barrier, leading to meningitis and shutdown of the central nervous system if left untreated (Ireton 2007).

Studying the mechanism of internalization via Internalin A as well as the factors that affect the success of *L. monocytogenes*'s entry into human cells is important because this research has important implications for drug delivery. The intestinal barrier is a highly selectively permeable interface that is responsible for delivering nutrients to the intestinal lumen and for barring the entry of pathogens. The integrity of the permeability of the intestinal barrier is extremely important in maintaining health. Although the intestinal barrier prevents pathogens from entering the intestine, it makes the transport and delivery of larger agents, such as proteins and capsulated particles, more difficult. This also hinders efficient delivery of drugs into the intestine ((Ashkan Farhadi 2003). However, certain pathogens are able to bind to mucins on the on the surface of mucosal epithelial cells, allowing them to permeate the intestinal barrier as well as internalize in epithelial cells in the intestine (Linden et al 2004). Taking advantage of the capabilities of Internalin A to successfully internalize in host cells is a starting point for improving the efficiency of drug delivery to the intestinal lumen.

CHAPTER 3

MATERIALS AND METHODS

pGEX-6p-1-InlA-1 Plasmid Purification

Prior to this experiment, the pGEX-6P-1-InlA-1 plasmid was obtained from Dr. Youn-Hi Woo, and was transformed into MAX Efficiency DH5αF'1Q *E. coli* competent cells (Invitrogen, Carlsbad, CA). Following transformation, the plasmid was purified using a Plasmid Purification Midi Kit (QIAGEN, Valencia, CA). Successful purification of the plasmid was confirmed by agarose gel electrophoresis.

BL21(DE3)pLysS Plasmid Transformation

Transformation of the plasmid into BL21(DE3)pLysS competent *E. coli* cells was performed according to the manufacturer's protocol. Two vials of cells were thawed, and only one of these two vials was transformed. 10 ng of the purified pGEX-6P-1-InlA-1 plasmid was added to one vial of cells, and the vial was incubated on ice for 30 minutes. The vials were then heat shocked for 30 seconds in a 42°C water bath and placed on ice. After heat shock, the vial was incubated at 37°C with 250 µL of S.O.C medium, provided with the cells, and shaken for one hour at 225 rpm. The transformed cells were then plated onto LB plates with 34 µg/mL chloramphenicol and 50 µg/mL ampicillin. The pLysS plasmid found in the BL21(DE3)pLysS cells endows the cells with chloramphenicol resistance, and the pGEX plasmid confers ampicillin resistance. Thus, competent *E. coli* cells transformed with the pGEX-6P-1-InlA-1 plasmid would be expected to grow successfully on plates containing chloramphenicol and ampicillin, whereas untransformed cells would not be able to grow on these plates. Untransformed BL21(DE3)pLysS cells were plated onto LB plates containing 34 µg/mL

chloramphenicol, in order to verify the functionality of the pLysS plasmid, as well as LB plates without antibiotics to test for phage contamination. The plates were then incubated overnight at 37°C.

Internalin A Gene Expression

Four colonies from the plate containing BL21(DE3)pLysS cells transformed with the pGEX-6P-1-InlA-1 plasmid were inoculated in 5 mL of LB media with 50 µg/mL ampicillin. This was done in order to select for successful transformants in the liquid cultures as well as the previously described plated transformation reactions. Four of these starter cultures were shaken overnight, at 37°C and 225 rpm. These cultures were then diluted in 250 mL of LB media and shaken again at 37°C and 225 rpm. Absorbance was monitored at 600 nm in order to find the mid-log phase of growth and determine the correct time to add IPTG to induce expression of the InlA gene in the cultures. When A_{600} was determined to be in the 0.4 to 0.6 range, IPTG was added and the cultures were shaken for another four hours. Further cell growth was stopped by placing the flasks on ice, and the cultures were then centrifuged for 30 minutes at 5000 rpm. Cell pellets were subsequently collected and stored at -20°C before protein purification.

Internalin A Protein Purification

Cell pellets were first lysed with B-PER Bacterial Protein Extraction Reagent, purchased from Pierce (Rockford, IL). Halt Protease Inhibitor Cocktail was purchased from Pierce (Rockford, IL) and added to the cell lysate at a concentration of 10 µL per mL of cell lysate. EDTA was also added at 10 µL per mL of cell lysate in order to inhibit metalloprotease activity. The cell pellets were weighed and then suspended in 4 mL of B-PER Bacterial Protein Extraction Reagent per gram of cell pellet. After incubating the

cell pellets with the B-PER Bacterial Protein Extraction Reagent for 15 minutes, the lysate was then centrifuged at 15000×g for 30 minutes in order to separate the soluble Internalin A-GST fusion protein from insoluble protein in the cell lysate. In this step, the insoluble protein was pelleted while Internalin A and other soluble protein was collected in the supernatant. The supernatant from this step was then combined with an equal amount of Equilibration/Wash Buffer and applied to 1 mL Glutathione Spin Columns. Both of these items were purchased from Pierce (Rockford, IL). Before applying the lysate to the columns, however, the GST spin purification columns were prepared by washing the resin with 2 mL of Equilibration/Wash buffer.

The cell lysate was incubated with the GST spin purification column for one hour before centrifugation. Unbound protein was collected in the flowthrough from centrifugation of the column and was washed off of the column using 1 resin bed volume of Equilibration/Wash buffer. The absorbance of the elute was monitored at 280 nm to confirm the presence of protein in solution. To equilibrate the column and facilitate effective binding of protease for the cleavage step, the column was washed with 1 resin bed volume (1 mL) of Cleavage Buffer, composed of 1 M PBS, 1 mM diothreithol (DTT) and 1 mM EDTA. 80 µL of Prescission Protease, purchased from GE, was combined with 920 µL Cleavage Buffer, applied to the column and shaken for four hours at 4° C. After incubation of the column with the protease, the column was then centrifuged in order to collect the protein of interest. At this point, we can expect the Prescission Protease to have successfully cleaved the bond between Internalin A and the attached GST tag, thus allowing us to elute Internalin A from the column by centrifugation and leaving the GST tag bound to the spin column. Subsequently, any Internalin A remaining

on the column was eluted with 1 resin bed volume of Equilibration/Wash buffer, and the absorbance of the elute was monitored at 280 nm to confirm the presence of protein in solution. GST was eluted from the column using Elution Buffer, consisting of 1 mL of 10X glutathione stock solution diluted in 9 mL of Equilibration/Wash Buffer.

SDS-PAGE and Coomassie Staining

Samples from steps in the protein purification protocol were analyzed using SDS-PAGE, in order to confirm the presence of Internalin A in solution. One Precise Protein Gel from Pierce (Rockford, IL) was inserted into a PAGE insert, which was then placed in a gel tank. The inner chamber and the gel tank were then filled with Tris-HEPES-SDS buffer. Samples were prepared by adding 5 µL Lane Marker Reducing Sample Buffer (Pierce, Rockford, IL) to 20 µL protein sample, denaturing the samples by heating them in 100°C water for 3-5 minutes, centrifuging the samples at 6300 rpm for 3 minutes and then collecting the supernatant. 10 µL of Spectra Multicolor Broad Range Protein Ladder (Pierce, Rockford, IL) were added to the first well. 25 µL of prepared samples from the elution of unbound proteins, the Cleavage Buffer elution step, the Equilibration/Wash buffer elution step, and the GST elution step were added to the next four wells. After loading the samples, the gel was run for 90 minutes at 110 V and 45 mA, until the dye front from the Lane Marker Reducing Sample Buffer was 0.5 cm away from the bottom of the gel.

After the run time was completed, the gel was washed three times with DI water to remove SDS, and was then stained with 20 mL of GelCode Blue Stain Reagent solution (Pierce, Rockford, IL) for 1 hour. The gel was then destained by shaking the gel

with DI water for 2 hours. Band intensity was monitored and the water was changed several times over the course of destaining.

Dialysis

In order to increase the concentration of protein in solution, the samples were dialyzed using Pierce 10K Molecular Weight Cutoff Protein Concentrators (Pierce, Rockford, IL). Concentrators with a molecular weight cutoff of 10000 Da were used because the molecular weight of InlA is 50 kDa, and the protein of interest must have more than twice the molecular weight of the molecular weight cutoff of the concentrators being used. The concentrator was inserted into a collection chamber and the membrane of the concentrator was rinsed with DI water. 500 µL of the protein sample was applied to the concentrator and centrifuged at 15000×g until the volume of protein sample in the concentrator was 100 µL. The retentate was then aspirated carefully from the concentrator using a 10 µL pipette, so as not to damage the concentrator membrane.

Bicinchoninic Acid Assay

The concentration of Internalin A in solution before and after dialysis was quantified by performing a BCA assay. DTT and glutathione are known reducing agents that interfere with the production of Cu^{1+} ions in the Biuret reaction, which are detected by bicinchoninic acid and cause the color change of solution in the presence of protein. Therefore, I used the Reducing Agent Compatible BCA Protein Assay Kit from Pierce (Rockford, IL). The concentration of protein in solution was determined by following the manufacturer's protocol. A Standard Control and Sample Control were prepared, and were differentiated by the presence of reducing agent. While the Sample Control was the same buffer as that of the protein samples, the Standard Control did not contain the

reducing agent and was used as a blank. BSA standards were diluted in the same buffer as the Standard Control (i.e. the same buffer as that of the samples without the reducing agent) to create protein solutions with concentrations ranging from 125 µg/mL to 2000 µg/mL BSA. The concentrations and volumes of diluent and BSA solution in each standard can be found in Table 1. 9 µL samples of Sample Control, Standard Control, BSA standard and Internalin A solution were added in a microplate to 4 µL of Compatibility Reagent Solution, which was provided with the kit in order to modify disulfide reducing agents to minimize interference with the assay. After shaking the microplate for one minute, the samples were incubated at 37°C for 15 minutes. After incubation, kit-provided Working Reagent was added to the samples in order to begin the process of detecting Cu^{1+} ions, and the samples were incubated at 37°C for 30 minutes. After cooling the microplate to room temperature, the absorbance of the samples, standards and controls was measured at 562 nm. A standard curve was generated by plotting the blank-corrected absorbance values for the standards against the concentrations of the standards in µg/mL, and was used to calculate the concentration of Internalin A in solution.

Labeling of Protein with FITC

While expressing and purifying the Internalin A protein, I simultaneously attempted to successfully bind FITC-labeled protein to microspheres in preparation for internalization studies with Caco-2 cells. In addition to InlA-conjugated microspheres and microspheres labeled only with FITC, protein-conjugated microspheres that could serve as a negative control would be required for internalization studies. Ovalbumin was used as a negative control because this protein has no internalization capabilities. Imject

Ovalbumin was purchased from Pierce (Rockford, IL) and labeled with FITC using the FluoReporter™ FITC Protein Labeling Kit from Life Technologies (Grand Island, NY). Ovalbumin was labeled following the manufacturer's protocol. After calculating the volume of reactive dye stock solution required to label the ovalbumin solution, a reaction tube was prepared by adding 20 µL of freshly prepared 1M sodium bicarbonate solution to 200 µL of ovalbumin solution. Immediately before beginning the labeling reaction, 37 µL of dimethylsulfoxide (DMSO) was added to the FITC dye stock solution. The labeling reaction was stirred for one hour and was kept protected from light.

After the labeling reaction was complete, FITC-OVA was then purified using spin columns provided in the labeling kit. The conjugate was first centrifuged in order to dispose of any precipitate, and was then loaded dropwise onto the spin column and centrifuged again. The resulting flowthrough was comprised of FITC-OVA suspended in approximately 250 µL of PBS and 2 mM sodium azide, in order to protect the conjugate from bacterial contamination. The concentration of protein and degree of labeling of the resultant conjugate was then calculated, and the conjugate was stored in aliquots at -20°C.

Conjugation of FITC-OVA to Microspheres

Immediately after labeling, FITC-OVA was bound to 2 µm and 0.5 µm carboxyl-terminated microspheres. The amount of protein required to form a monolayer on the microspheres was calculated based on the density and diameter of the microspheres as well as the capacity of the microsphere surface to bind the protein. The capacity of the microsphere surface to bind a particular protein is a constant that is related to the molecular weight of the protein. Capacities for two proteins, bovine serum albumin (BSA) and bovine IgG (BIgG), were found to be 3 mg/m^2 and 2.5 mg/m^2 respectively.

The molecular weight of BSA is 65 kDa, whereas the molecular weight of BIgG is 150 kDa. The molecular weight of ovalbumin, which is 45 kDa, is more comparable to the molecular weight of BSA than that of BIgG. Therefore, the capacity of the microspheres to bind ovalbumin was approximated to be equal to 3 mg/m^2.

Subsequently, the mass of microspheres per microliter of microsphere suspension was calculated by using the percent solid, and this mass was then used to calculate the total mass of protein needed to saturate the microspheres. It is recommended that a 3-10X excess of the calculated mass of protein required to form a monolayer is incubated with the microspheres in order to increase specificity of binding and to make sure the protein is correctly spatially oriented. Therefore, a fourfold excess of protein was covalently coupled to 50 µL of 2 µm microbeads, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS). 50 µL of microbeads were diluted with 950 µL of MilliQ water. After centrifugation at 3600 rpm for 15 minutes, the supernatant was removed and the beads were resuspended in 0.1 M MES 0.8% NaCl buffer with a pH of 4.7. 2-(N-morpholino) ethanesulfonic acid (MES) was used as an activating agent in order to increase efficiency of coupling of the labeled protein to the microbeads. 1 mg of EDC and 1 mg of Sulfo-NHS were added to the bead suspension, incubated at room temperature for 20 minutes and centrifuged again. After resuspension of the bead solution in water and subsequent centrifugation, the beads were resuspended in PBS with 11.9 µL of FITC-OVA and incubated for two hours. Subsequently, any unbound protein was discarded by washing and centrifuging the beads three times with 1 mL of PBS.

CHAPTER 4
RESULTS

BL21(DE3)pLysS Transformation

10 ng of the purified pGEX-6P-1-InlA-1 plasmid was transformed into one vial of BL21(DE3)pLysS cells. This transformation reaction was then plated in 50 µL and 150 µL volumes onto two LB plates containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol in order to select for viable transformants expressing the pLysS plasmid, which confers chloramphenicol resistance, as well as the pGEX-6P-1-InlA-1 plasmid, which confers ampicillin resistance. White colonies were observed on both of these plates, confirming viability of the BL21 cells as well as successful transformation with the pGEX-6P-1-InlA-1 plasmid. As expected greater number of colonies were observed on the 150 µL plate than on the 50 µL plate. However, the colonies on the 50 µL plate were larger in diameter than the colonies on the 150 µL plate, as portrayed in Figure 1A and 1B.

Untransformed BL21(DE3)pLysS cells were plated onto three LB plates: one that did not contain antibiotics in order to verify a lack of phage contamination, one with 50 µg/mL ampicillin in order to verify a lack of ampicillin resistance contamination, and one with 34 µg/mL chloramphenicol in order to verify viability of the cells and expression of the pLysS plasmid. Lawn growth was observed on the plates containing 34 µg/mL chloramphenicol and no antibiotics, and no growth was observed on the plate containing 50 µg/mL ampicillin, as demonstrated in Figure 1C, 1D and 1E.

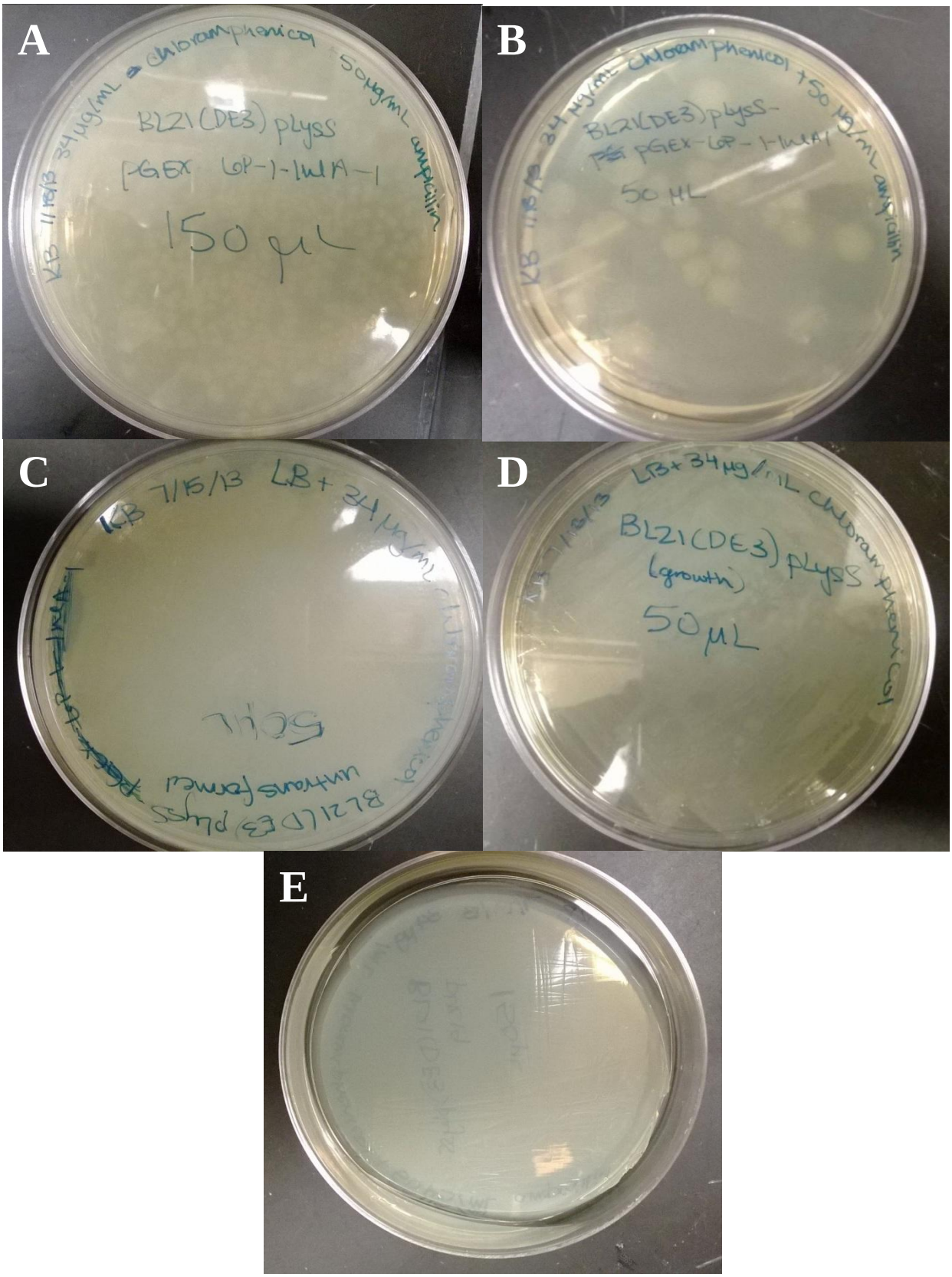

Figure 1. Plating of BL21(DE3)pLysS cells. A) 150 µL transformed BL21(DE3)pLysS cells. B) 50 µL transformed BL21(DE3)pLysS cells. C) 50 µL untransformed BL21(DE3)pLysS cells on LB without antibiotics. D) 50 µL untransformed BL21(DE3)pLysS cells on LB with 34 µg/mL chloramphenicol. E) 50 µL untransformed BL21(DE3)pLysS cells on LB with 50 µg/mL ampicillin.

SDS-PAGE

SDS-PAGE was conducted in order to verify expression of the pGEX-6P-1-InlA-1 plasmid as well as the presence of purified InlA in solution.

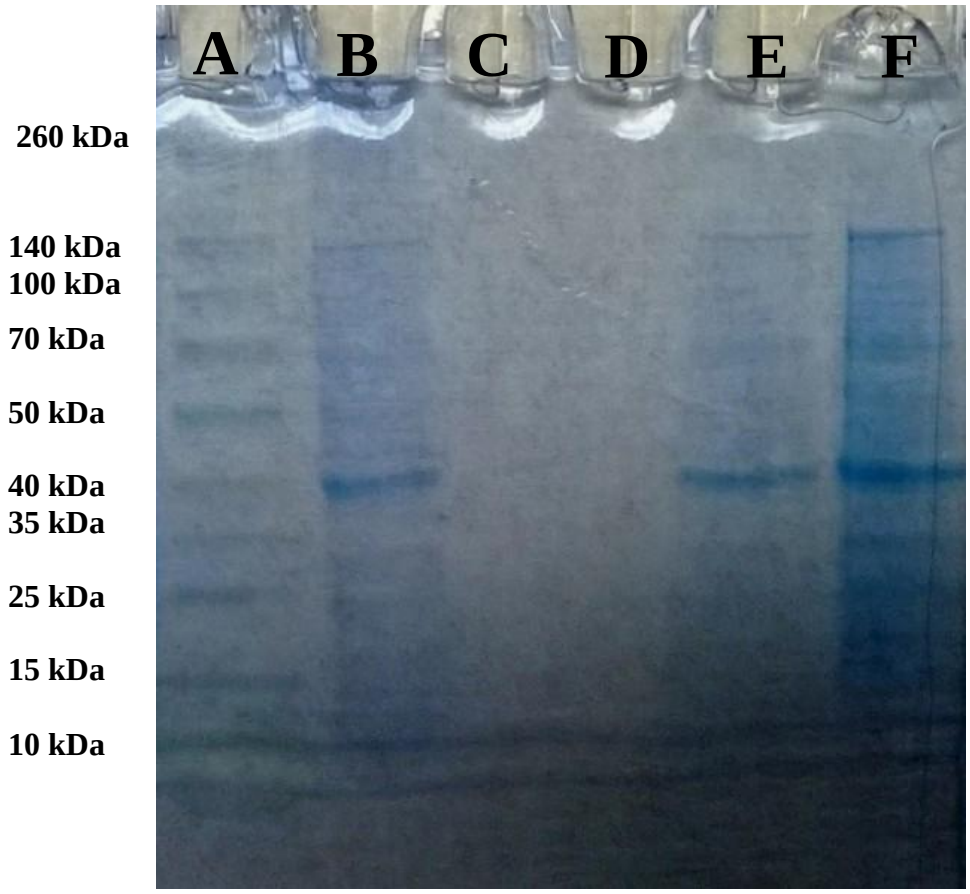

Figure 2. SDS-PAGE of samples from protein purification. Well A) Spectra Multicolor Broad Range Protein Ladder (Pierce). Well B) Unbound protein from transformed BL21(DE3)pLysS cell lysate in Equilibration/Wash buffer elute. A weak 75 kDa band can be seen in this sample, as well as weak protein bands ranging from 10 kDa to 260 kDa. A very strong 40 kDa band is also present in this sample. Well C) Cleavage Buffer elution of InlA using Prescission Protease. The absence of bands in this well indicates that no protein was eluted in this wash. Well D) Equilibration/Wash buffer elution of remaining InlA. The absence of bands in this well indicates that no protein was eluted in this wash. Well E) Elution Buffer wash step. In this step, GST was eluted from the column with glutathione. A 75 kDa and 40 kDa band were detected in this elute. The 75 kDa band is slightly stronger than that in Well B. Background protein bands are also observable, but are weaker than those in Well B. Well F) Elution Buffer wash solution after dialysis. All protein bands observed in Well E are stronger in Well F, indicating successful concentration of the protein solution.

BCA Assay

BSA standards were created by serial dilutions using the volumes of diluents and BSA described in Table 1, in order to generate standards with concentrations ranging from 125 µg/mL to 2000 µg/mL. A standard curve was generated by plotting the corrected A_{562} against protein concentration (Figure 2).

Vial	Diluent Volume (µL)	BSA Source/Volume (µL)	Concentration (µg/mL)
A	0	200	2000
B	66	200	1500
C	100	100	1000
D	100	100	750
E	100	100	500
F	100	100	250
G	100	100	125

Table 1. Serial dilutions used to create BSA standards

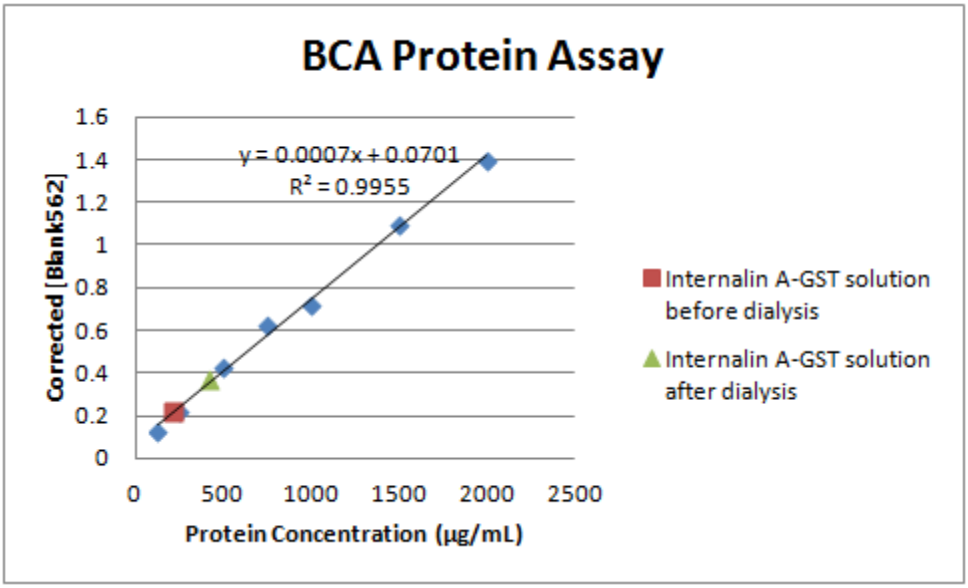

Figure 3. BCA assay standard curve generated by plotting corrected A_{562} against protein solution concentration. The line of best fit associated with the curve has a coefficient of determination of 0.9955.

Because the presence of Internalin A-GST fusion protein in the GST elution step was confirmed by SDS-PAGE, the equation of the line of best fit was used to calculate the concentration of protein in this elute before and after dialysis. A_{562} of the Elution Buffer elute before dialysis was found to be 0.261, and A_{562} of the Elution Buffer elute after dialysis was found to be 0.454. The concentration of protein in solution before dialysis was calculated to be 272.7 µg/mL (Equation 1) and the concentration of protein in solution after dialysis was 548.4 µg/mL (Equation 2).

$$0.261 = 0.0007x + 0.0701$$

Equation 1. Calculation of concentration of protein in the Elution Buffer elute before dialysis. The concentration of protein in solution was calculated to be 272.7 µg/mL.

$$0.454 = 0.0007x + 0.0701$$

Equation 2. Calculation of concentration of protein in the Elution Buffer elute after dialysis. The concentration of protein in solution was calculated to be 548.4 µg/mL.

Labeling of Ovalbumin with FITC

After labeling ovalbumin with FITC, the concentration of protein in solution was calculated by measuring the absorbance of the protein conjugate at 494 nm and 280 nm (Equation 3). Absorbance of the conjugate was measured at 494 nm because this is the maximum absorption wavelength for FITC. The molarity of the protein solution was subsequently used to calculate the degree of labeling of the protein (Equation 4).

$$[\text{FITC}-\text{OVA}](\text{M}) = \frac{[A_{280}-(A_{494}\times 0.3)]\times \text{dilution factor}}{\varepsilon} = \frac{[2.723-(2.616\times 0.3)]\times 4}{24000} = 0.000323\ \text{M}$$

Equation 3. Calculation of final concentration of FITC-OVA in solution. The molar extinction coefficient of ovalbumin is denoted by $\boldsymbol{\varepsilon}$ and was given to be 24000. 0.3 is a correction factor used to correct the A_{494} reading for the absorption of FITC at 280 nm.

$$\text{molecules of dye per protein molecule} = \frac{A_{494}\ \times \text{dilution factor}}{68000 \times [\text{FITC}-\text{OVA}]}$$
$$= \frac{2.616 \times 4}{68000 \times 0.000323} = 0.48$$

Equation 4. Calculation of degree of labeling of protein. The molar extinction coefficient of FITC at 494 nm is 68000 $cm^{-1}M^{-1}$.

Conjugation of FITC-OVA to Microspheres

The mass of protein required to form a monolayer on 1 gram of polystyrene-based microspheres was calculated based on the density and diameter of the microspheres as well as the capacity of the microsphere surface to bind the protein (Equation 3). The density of polystyrene is 1.05 g/cm^3, and the capacity of the microspheres to bind ovalbumin was estimated to be 3 mg/m^2 (Bangs Laboratories). Using these constants, the mass of protein required to saturate 1 gram of 2 µm microspheres was calculated to be 8.57 mg. The percent solid of the microspheres was 10.05%. This percent solid was used to calculate the total mass of spheres present in 50 µL of suspended microspheres and the mass of protein needed to saturate the microspheres was calculated to be 0.043 mg (Equation 4). Finally, a fourfold excess of protein was incubated with the microspheres during conjugation, and the volume of protein solution required was determined to be 11.9 µL (Equation 5). Successfully covalent coupling of FITC-OVA to the microspheres can be observed in Figure 4.

$$S = \left(\frac{6}{\rho\, D}\right)(C)$$

Equation 5. Mass of protein required to saturate one gram of 2 µm microspheres. In this equation, ρ represents the density of the microspheres, D represents the diameter of the microspheres in micrometers, and C represents the capacity of the microsphere surface to bind the protein.

$$\frac{0.1005\ \text{mg beads}}{\mu\ \text{L}} \times 50\ \mu\ \text{L} \times \frac{1\ \text{g}}{1000\ \text{mg}} \times \frac{8.57\ \text{mg protein}}{\text{g beads}} = 0.043\ \text{mg FITC} - \text{OVA}$$

Equation 6. Mass of FITC-OVA required to saturate 50 µL of 2 µm microspheres.

$$0.043\ \text{mg FITC} - \text{OVA} \times 4 \times \frac{1\ \text{mL}}{14.5\ \text{mg FITC} - \text{OVA}} \times \frac{1000\ \mu\text{L}}{\text{mL}} = 11.9\ \mu\text{L FITC} - \text{OVA}$$

Equation 7. Volume of FITC-OVA needed to saturate 50 µL of 2 µm microspheres.

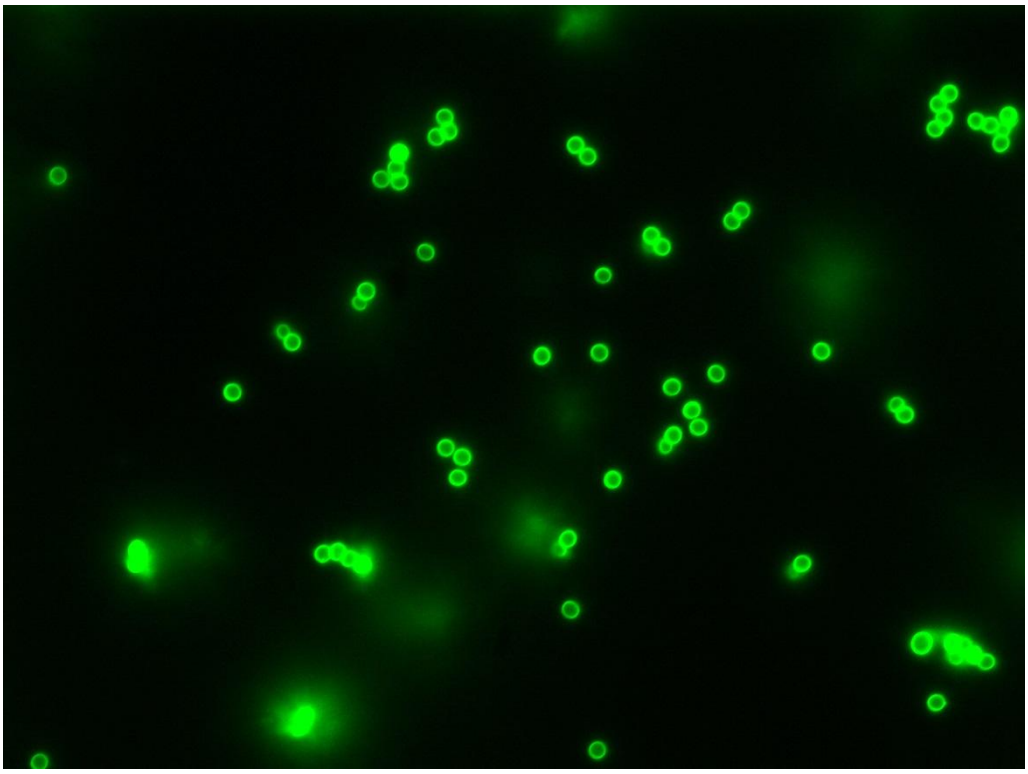

Figure 4. 2 µm carboxyl-terminated polysterene microspheres conjugated with FITC-OVA.

CHAPTER 5

DISCUSSION

The use of BL21(DE3)pLysS *E. coli* competent cells affords us the ability to control the timing of gene expression because of the presence of the DE3 lysogen in these cells. The DE3 lysogen carries the gene for T7 RNA polymerase, whose expression is regulated by the *lac*UV5 promoter. Transcription of the *lac*UV5 promoter is inducible in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG). In this way, protein yield can be maximized by inducing transcription of T7 RNA polymerase during the mid-log phase of exponential bacterial growth. Lawn growth of untransformed cells and an absence of clear patches were observed in the absence of antibiotics, thus confirming a lack of phage contamination of the cells. Lawn growth of untransformed cells was also observed in the presence of chloramphenicol. Because the pLysS plasmid confers chloramphenicol resistance, untransformed cells are expected to grow in the presence of chloramphenicol, and this plate confirms the integrity of the pLysS plasmid. The absence of growth observed in the presence of ampicillin confirms a lack of ampicillin resistant contamination. The pLysS plasmid does not confer ampicillin resistance; thus, growth of BL21(DE3)pLysS cells would not have been expected unless they had been transformed with a plasmid that does confer ampicillin resistance. Successful transformation of the BL21(DE3)pLysS cells with the pGEX-6P-1-InlA-1 plasmid was confirmed by observing growth of the transformed cells in the presence of both chloramphenicol and ampicillin. However, colonies formed by transformed cells on the 50 µL plate were larger than those formed by the transformed cells on the 150 µL plate. This disparity in size may be due to

the fact that the plates were taken out of the incubator and stored at 4°C earlier than recommended.

After purification of the protein, expression of the InlA-GST fusion protein was confirmed by the 75 kDa band observed in the Elution Buffer elute from the GST elution step. The molecular weight of InlA is 50 kDa, and the molecular weight of GST is 25 kDa. Thus, this confirms that the fusion protein is still intact and was not successfully cleaved with the PreScission Protease. In addition, no protein was eluted during the Cleavage Buffer elution step or the subsequent Equilibration/Wash buffer elution step, also indicating that the protease did not cleave the bond between the GST tag and InlA, and that the fusion protein was still bound to the column before elution with glutathione. This means that after the column was washed with Elution Buffer, the excess glutathione in the Elution Buffer led to washing of the GST off of the column, resulting in elution of the entire fusion protein in this step.

In order to increase the concentration of InlA-GST fusion protein in solution, the Elution Buffer elute was dialyzed using Pierce Protein Concentrators with a molecular weight cutoff of 10000 Da. A BCA assay was performed to calculate the concentration of protein in solution. Because the concentration of protein in solution is higher in the protein sample after dialysis than before dialysis, dialysis was successful. However, its efficiency could have been improved because in the SDS-PAGE fraction of the dialyzed protein sample, a strong 40 kDa protein band was observed in solution. This is because the 40 kDa fragment has a molecular weight of more than twice the molecular weight cutoff of the protein concentrator and was thus retained in the concentrator rather than being filtered out. However, by aiming to dialyze the InlA-GST fusion protein and

cleave the bond between the GST tag and InlA in solution rather than elute InlA off of the glutathione spin column with the protease, the molecular weight cutoff of the protein concentrators used could be increased. Because the molecular weight of InlA-GST is 75 kDa, protein concentrators with a molecular weight cutoff of 30 kDa could be used instead, thus increasing the specificity of dialysis and increasing the purity of the protein in solution.

Ovalbumin was chosen as a negative control because its molecular weight is comparable to that of InlA. Therefore, ovalbumin and InlA have comparable adsorption capacities to polystyrene microbeads, but ovalbumin is a storage protein, and it is therefore unlikely that it would be involved in receptor mediated entry. I chose to label ovalbumin with FITC before coupling the protein to microspheres in order to be able to observe coupling of the protein to the microspheres through fluorescent microscopy. Because the uncoupled polystyrene beads do not have inherent fluorescence, the presence of fluorescence on the beads indicates successful coupling of protein to the microspheres. In addition, there is very little fluorescence in the buffer surrounding the microspheres, indicating that unbound FITC-OVA was successfully washed off of the microspheres. The degree of labeling of the ovalbumin solution was found to be 0.48 molecules of FITC per protein molecule. The optimal degree of labeling is 4-8 molecules of FITC per protein molecule (Invitrogen). However, this value is optimal for labeling of antibodies, which have significantly higher molecular weights than ovalbumin. While the optimal degree of labeling for ovalbumin is very likely smaller than the value recommended by Invitrogen, a future step for optimizing this protocol would be to perform labeling reactions with varied molar ratios of dye to

protein, in order to find the optimal molar ratio of dye to protein that should be used when labeling ovalbumin.

Even though Internalin A has not been successfully purified yet, the described protocol for labeling ovalbumin with FITC and binding FITC-OVA to microspheres can be used for Internalin A as well. This is because the capacity of the microsphere to bind a particular protein is a principal factor in determining the mass of protein required to saturate 1 gram of microspheres, and varies based on the molecular weight of the protein in question. The capacity of polystyrene microspheres to bind ovalbumin was estimated based on the molecular weight of BSA, which is 65 kDa. The molecular weight of ovalbumin is 45 kDa, and is comparable to that of InlA, which is 50 kDa.

Future steps for this project include eluting the InlA-GST fusion protein and incubating the fusion protein with PreScission Protease in solution in order to verify functionality of the protease, rather than applying the protease to the column. The first step required is to successfully purify the InlA protein. After functionalizing microspheres with FITC-labeled and purified InlA, the microspheres will be incubated with Caco-2 cells, which have already been obtained from Dr. Andrew Neish's lab. Internalization efficiency will be observed in InlA-functionalized microspheres and compared to ovalbumin-functionalized microspheres and microspheres conjugated only with FITC, in order to elucidate the effect of the presence of InlA on entry of microspheres into human cells. The effect of ligand density and bead size on internalization of microspheres will also be observed using fluorescent microscopy and flow cytometry. By performing internalization studies and observing success of Internalin A-mediated entry of microspheres in various epithelial cell lines, we can

progress towards creating a more efficient system of drug delivery to the intestinal lumen. Using receptor-mediated internalization to deliver drugs to the intestinal epithelium could increase the rate of success with which drugs are able to cross the selectively permeable barrier of the intestinal epithelium, thus increasing efficiency and success of treatment with particular drugs and possibly reducing the dosage required for treatment.

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